Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of Arabidopsis FLS2 exhibiting characteristically different perception specificities

Robatzek, Silke ; Bittel, Pascal ; Chinchilla, Delphine ; Köchner, Petra ; Felix, Georg ; Shiu, Shin-Han ; Boller, Thomas

Abstract: Bacterial flagellin is known to stimulate host immune responses in mammals and plants. In Arabidopsis thaliana, the receptor kinase FLS2 mediates flagellin perception through physical interaction with a highly conserved epitope in the N-terminus of flagellin, represented by the peptide flg22 derived from Pseudomonas syringae. The peptide flg22 is highly active as an elicitor in many plant species. In contrast, a shortened version of the same epitope derived from Escherichia coli, flg15E coli, is highly active as an elicitor in tomato but not in A. thaliana or Nicotiana benthamiana. Here, we make use of these species-specific differences in flagellin perception abilities to identify LeFLS2 as the flagellin receptor in tomato. LeFLS2 is most closely related to AtFLS2, indicating that it may represent the flagellin receptor of tomato. Expression of the LeFLS2 gene in Arabidopsis did not result in accumulation of its corresponding gene product, as indicated by experiments with LeFLS2-GFP fusions. In contrast, expression of LeFLS2-GFP fusions in N. benthamiana, a species that, like tomato, belongs to the Solanaceae, was obviously functional. N. benthamiana plants transiently expressing a LeFLS2-GFP fusion acquired responsiveness to flg15E coli to which they are normally unresponsive. Thus, LeFLS2 encodes a functional, specific flagellin receptor, the first to be identified in a plant family other than the Brassicaceae

DOI: https://doi.org/10.1007/s11103-007-9173-8

Plant Mol Biol (2007) 64:539–547
DOI 10.1007/s11103-007-9173-8

Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of *Arabidopsis* FLS2 exhibiting characteristically different perception specificities

Silke Robatzek · Pascal Bittel · Delphine Chinchilla · Petra Köchner · Georg Felix · Shin-Han Shiu · Thomas Boller

Received: 17 November 2006 / Accepted: 14 April 2007 / Published online: 25 May 2007

Abstract Bacterial flagellin is known to stimulate host immune responses in mammals and plants. In *Arabidopsis thaliana*, the receptor kinase FLS2 mediates flagellin perception through physical interaction with a highly conserved epitope in the N-terminus of flagellin, represented by the peptide flg22 derived from *Pseudomonas syringae*. The peptide flg22 is highly active as an elicitor in many plant species. In contrast, a shortened version of the same epitope derived from *Escherichia coli*, flg15$^{E\ coli}$, is highly active as an elicitor in tomato but not in *A. thaliana* or *Nicotiana benthamiana*. Here, we make use of these species-specific differences in flagellin perception abilities to identify LeFLS2 as the flagellin receptor in tomato. LeFLS2 is most closely related to AtFLS2, indicating that it may represent the flagellin receptor of tomato. Expression of the *LeFLS2* gene in *Arabidopsis* did not result in accumulation of its corresponding gene product, as indicated by experiments with LeFLS2-GFP fusions. In contrast, expression of LeFLS2-GFP fusions in *N. benthamiana*, a species that, like tomato, belongs to the *Solanaceae*, was obviously functional. *N. benthamiana* plants transiently expressing a *LeFLS2-GFP* fusion acquired responsiveness to flg15$^{E\ coli}$ to which they are normally unresponsive. Thus, *LeFLS2* encodes a functional, specific flagellin receptor, the first to be identified in a plant family other than the *Brassicaceae*.

Keywords FLS2 homologue · flg22 · Immunity · PAMP · *Solanaceae*

Abbreviations

At	*Arabidopsis thaliana*
BAK1	BRI1-associated kinase 1
BRI1	Brassinosteroid Insensitive 1
CDNA	Complementary DNA
CLV1	CLAVATA 1
Col-0	Columbia-0
E. coli	*Escherichia coli*
EFR	EF-Tu receptor
EF-Tu	Elogation factor Tu
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
flg22	Flagellin elicitor-active epitope
FLS2	Flagellin Sensing 2
GFP	Green fluorescent protein
GUS	β-Glucoronidase
Le	*Lycopersicum esculentum*
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern

Electronic supplementary material The online version of this article (doi:10.1007/s11103-007-9173-8) contains supplementary material, which is available to authorized users.

S. Robatzek · P. Bittel · D. Chinchilla · G. Felix · T. Boller
Zürich-Basel Plant Science Center, Botanical Institute, University Basel, Hebelstrasse 1, Basel 4056, Switzerland

S. Robatzek (✉) · P. Köchner
Department of Plant-Microbe Interactions, Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, Cologne 50829, Germany
e-mail: robatzek@mpiz-koeln.mpg.de

G. Felix
ZMBP, University of Tübingen, Auf der Morgenstelle 5, Tübingen 72076, Germany

S.-H. Shiu
Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA

PRR	Pattern recognition receptor
P. syringae	*Pseudomonas syringae*
RACE	Rapid amplification of cDNA ends
RLK	Receptor-like kinase
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
TLR	Toll-like receptor
Ws-0	Wassilewskaja-0

Introduction

Plants, like animals, contain a sophisticated array of immune receptors to distinguish between self and non-self (Medzhitov and Janeway 2002; Akira and Takeda 2004; Chisholm et al. 2006). A family of pattern recognition receptors (PRRs) enables the plant basal innate immune system to sense diverse microbial pathogen-associated molecular patterns (PAMPs) such as fungal xylanase, bacterial flagellin and EF-Tu (Felix et al. 1999; Kunze et al. 2004; Ron and Avni 2004). PAMP perception triggers numerous immune responses including the rapid production of reactive oxygen species (ROS), ethylene and changes in gene expression (Felix et al. 1999; Kunze et al. 2004; Zipfel et al. 2004), which mostly exhibit a transient behavior. Downstream PAMP responses include the accumulation of antimicrobial metabolites and cell wall alterations such as callose deposition (Gomez-Gomez et al. 1999; Zipfel et al. 2004). PAMP signaling pathways involve the rapid and transient activation of a mitogen-activated protein (MAP) kinase cascade (Nuhse et al. 2000; Asai et al. 2002; Zipfel et al. 2006).

In mammals, flagellin induces inflammatory responses through Toll-like receptor 5 (TLR5). Monomeric flagellin binds directly to TLR5, which is mediated through a domain formed by a conserved N- and C-terminal part of the protein (Smith et al. 2003). In contrast, plants respond to a single amino acid stretch covering the most conserved region in the N-terminus of the flagellin protein (Felix et al. 1999). A 22 amino acid peptide spanning this region in the flagellin of *Pseudomonas syringae*, flg22, is sufficient to elicit the whole array of typical immune responses in a broad variety of plants. Comparative studies between the *Brassicaceae Arabidopsis thaliana* and the *Solanaceae* tomato showed that both hold a highly sensitive perception system for flg22, but also revealed distinct specificities for variants of this peptide. Whereas the peptide flg15, a N-terminally shortened version of flg22, elicits responses only at higher concentrations in *A. thaliana*, it is highly active in tomato (Meindl et al. 2000; Bauer et al. 2001; Chinchilla et al. 2006). This discrimination between peptide requirements in *A. thaliana* and tomato is even more pronounced with a flg15 variant corresponding to the flagellin sequence of *E. coli*, $flg15^{E.coli}$ (Meindl et al. 2000; Bauer et al. 2001).

In *A. thaliana*, the receptor for flagellin is encoded by the *FLS2* gene (*Flagellin Sensing 2*). Mutant plants lacking the *FLS2* receptor are insensitive to flg22 (Gomez-Gomez and Boller 2000), are impaired in flg22 binding (Gomez-Gomez et al. 2001), and they show enhanced susceptibility to infection with pathogenic bacteria (Zipfel et al. 2004). *FLS2* codes for a leucine-rich repeat (LRR) receptor serine/threonine kinase with a single transmembrane region. FLS2 is localized at the plant plasma membrane and undergoes endocytosis upon stimulation (Robatzek et al. 2006). It physically interacts with flg22, as shown by cross-linking experiments; in addition, upon heterologous expression of FLS2, tomato cells gained the flagellin perception system characteristic for *A. thaliana* (Chinchilla et al. 2006).

Receptor-like kinases (RLK) form a large gene family with more than 600 members in *A. thaliana*, and the LRR-type RLKs represent the largest subgroup with about 200 (Shiu and Bleecker 2001). Functionally only few of them have been identified, of which e.g. CLAVATA1 (CLV1) plays a role in meristem development, Brassinosteroid Insensitive 1 (BRI1) and BRI1-associated kinase (BAK1) mediate perception of the plant hormone brassinosteroid, and FLS2 as well as the recently identified EF-Tu receptor (EFR) are involved in non-self detection (Dievart and Clark 2004; Zipfel et al. 2004; Vert et al. 2005; Zipfel et al. 2006). Additional insights into their functions can be provided by comparative analysis of homologous receptors in another species. For example, the tomato RLK SR160 has been postulated to exhibit dual functionality as receptor for brassinosteroid and systemin (Montoya et al. 2002).

In this study, we aimed at identifying and functionally characterizing the corresponding flagellin receptor in tomato, especially because of its distinct ligand specificities compared to *Arabidopsis* FLS2. We amplified a sequence from both, tomato cell cultures and tomato plants that displayed high amino acid sequence homology to AtFLS2. Expression of *LeFLS2* under the control of the *AtFLS2* promoter did not lead to LeFLS2 protein accumulation in *A. thaliana*. However, we achieved expression of LeFLS2 fused to the green fluorescent protein (GFP) in the more closely related *solanaceous* species *Nicotiana benthamina*. Exploiting species-specific differences within the flagellin perception systems of tomato and *N. benthamiana* we demonstrate that *LeFLS2* encodes the tomato flagellin receptor, the first functionally characterized AtFLS2 ortholog.

Materials and methods

Cloning and construction of LeFLS2

FLS2 sequences were aligned against plant EST assemblies from MIPS and plant EST sequences from GenBank. Best matches to *Arabidopsis FLS2* and rice sequences as well as phylogenetic analysis of the kinase domains identified EST273998 (GI5889499) as the closest homologue from tomato. Poly-A-RNA was extracted from tomato Msk8 cell cultures (Meindl et al. 2000), and tomato plants variety ''Roter Gnom'' (Syngenta), and used for cDNA synthesis (Ambion). Nested primer pairs 5′-AAT CCT GTT GGA CAA CGT CCA GTC ATC CTC-3′ and 5′-CAG ATC CAA GTG CTG CAA GTA TGA TCC AGG-3′, 5′-CCT GGA TCA TAC TTG CAG CAC TTG GAT CTG-3′ and 5′-GAG GAT GAC TGG ACG TTG TCC AAC AGG ATT G-3′ for 5′- and 3′-RACE, respectively were designed based on the EST273998. 5′- and 3′-RACE sequences were amplified using the RLM-RACE kit (Ambion), products were cloned into TOPO vector (Invitrogen) and sequenced. Full length *LeFLS2* was amplified on genomic DNA extracted from tomato plants using Expand High Fidelity Taq Polymerase (Roche) with primers 5′-TCT TTT GAA ATG ATG ATG TTA AAG ACA GTT G-3′ and SalI-containing 5′-TTA AGT CGA CTA ATC TTT TAC CAA ATG AGA AGG CAT AC-3′. The genomic *LeFLS2* product was introduced into the pCAMBIA2300-*FLS2p::FLS2-3myc-GFP* (Robatzek et al. 2006) construct by replacing *FLS2–3myc-GFP* with *LeFLS2* at blunted BamHI and SalI restriction sites. For GFP tagging the *LeFLS2* stop codon was mutated by PCR with the SalI-containing primer 5′-TTA AGT CGA CTT GAT CTT TTA CCA AAT GAG AAG GCA TAC-3′, and GFP was subsequently cloned in-frame into the SalI site. All sequencing was done at Microsynth. Deduced protein sequence analysis and alignments were performed using Pileup of the GCG software package, Box Shade from at http://www.ch.embnet.org/software/Box_form.html; protein motif inspection at http://searchlauncher.bcm.tmc.edu/

http://homepage.univie.ac.at/herbert.mayer/Main-PROT.html

http://www.expasy.ch

http://dbptm.mbc.nctu.edu.tw/prediction.html

Plant transformation and analysis

A *FLS2p::LeFLS2-GFP* construct was used to transform *A. thaliana* Ws-0 (Ws/LeFLS2-GFP) and *N. benthamiana* leaves. Controls were *FLS2p::FLS2-GFP*, *SIRKp*::GUS constructs, and the Ws/FLS2-GFP transgenic line (Zipfel et al. 2004; Robatzek et al. 2006). Stable transgenic lines were generated using the standard *A. tumefaciens*-mediated gene transfer procedure of inflorescence infiltration. Independent transformed plant pools were kept separate for selection of independent transgenic lines based on their kanamycine resistance. Plants of T2 and T3 generation were chosen for experiments. To assay transgene expression, RNA extraction was done using the Plant RNeasy kit (Qiagen), and RT-PCR was performed with primer pairs 5′-CCT GGA TCA TAC TTG CAG CAC TTG GAT CTG-3′ and 5′-CAA TCC TGT TGG ACA ACG TCC AGT CAT CCT C-3′ (LeFLS2), and 5′-TTG TGC ACC GAG CCA TCG TG-3′ and 5′-GAG AGT GAG AAG ATG TAC TC-3′ (RPL4) using the RT-PlusPCR kit (Eppendorf). Western-blot analysis was done on crude membrane extracts of liquid grown seedlings using a polyclonal anti-GFP rabbit serum (Molecular Probes).

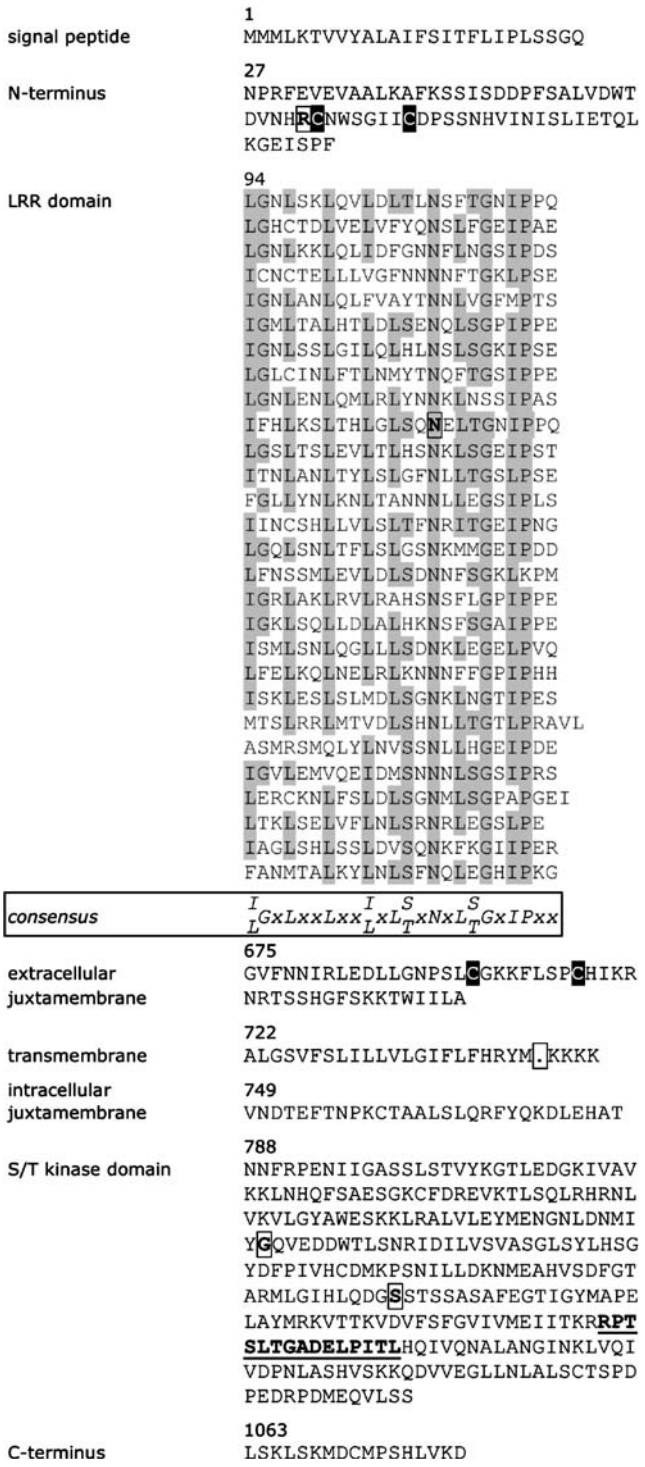
Transient transformation of *N. benthamiana* was performed as described (Zipfel et al. 2006). Western blot analysis and cross-linking experiments were done on crude membrane extracts of pooled transformed leaves according to Zipfel et al. 2006.

Functional complementation by LeFLS2-GFP was assayed in standard procedures measuring chemical binding, oxidative burst, ethylene production, and seedling fresh weight (Felix et al. 1999; Gomez-Gomez et al. 1999; Bauer et al. 2001). Flagellin peptides were used with following sequences: flg22 - QRL STG SRI NSA KDD AAG LQI A, flg15 - RIN SAK DDA AGL QIA, and flg15$^{E.coli}$ - RIN SAK DDA AGQ AIA.

Results

Identification and cloning of LeFLS2

Tomato is highly responsive to flagellin peptides in a characteristic manner. Biochemical cross-linking experiments with radiolabeled flg22 in tomato cells revealed a specific band at ~175 kDa, which is similar to the previously described *Arabidopsis* flagellin binding site (Suppl. Fig. 1). Using BLAST programs we screened available tomato EST databases, and we found EST273998 (GI5889499) from *Lycopersicum esculentum* (recently renamed *Solanum lycopersicum*) to exhibit striking homology to *AtFLS2*. Starting from this EST sequence we performed 5′- and 3′-RACE on RNA derived from either tomato cell culture (Msk8) or tomato plants (*L. esculentum*). The sequences obtained from the respective RACE products were used to amplify a full-length sequence from genomic DNA. The gene called *LeFLS2* was found to encode a transmembrane LRR receptor kinase of 1170 amino acid with a calculated mass of approximately 130 kDa (Fig. 1). LeFLS2 has a predicted signal peptide, and on its extracellular part a LRR domain consisting of 28 tandem



repeats with the plant characteristic consensus sequence *LGxLxxLxxLxLSxNxLSGxIPxx* (Kobe and Kajava 2001). Adjacent to the LRR domain is a region with paired cysteines typical for LRR receptor kinases (Dievart and Clark 2003), followed by a single transmembrane domain, an intracellular juxtamembrane region, and a serine/threonine kinase domain (Walker 1994). Similarly to AtFLS2, the

◀**Fig. 1** The LRR receptor kinase LeFLS2. Deduced amino acid sequence of the *LeFLS2* genomic clone from tomato plants. The predicted signal peptide, the N-terminal domain, the LRR domain and its derived LRR consensus, the extracellular juxtamembrane region, the single transmembrane domain, the intracellular juxtamembrane region, the serine/threonine (S/T) kinase, and the C-terminal tail are indicated. Paired cysteine residues are highlighted in black boxes, residues matching the LRR consensus are marked in gray boxes, the predicted PEST-like motif is underlined in bold, and residues differing/lacking between LeFLS2 from tomato plants and cell culture are boxed

LeFLS2 kinase contains a PEST-like motif in its C-terminal part, which is a polypeptide sequence enriched in proline, glutamate, serine, and threonine that may mediate attachment of ubiquitin molecules targeting proteins for degradation (Rechsteiner and Rogers 1996). The *LeFLS2* ortholog from Msk8 cell cultures, a hybrid derived from *L. esculentum* and *L. peruvianum* was also cloned. Comparison of the respective *LeFLS2* sequences revealed five amino acid differences in the following positions (tomato plant/Msk8 cells): 61R/H, 324N/Y, 744-/K, 879G/C, and 949S/G), which might reflect differences between *L. esculentum* and *L. peruvianum* (Felix et al. 1991).

Sequence alignments of AtFLS2, LeFLS2, and the receptor for bacterial EF-Tu, AtEFR (Zipfel et al. 2006), were performed. The AtFLS2 and LeFLS2 LRR domains share 55% amino acid identity (Suppl. Fig. 2), their respective kinase domains 59% (data not shown). Interestingly, lowest homology was found in a region stretching from LRR-2 to LRR-5. For comparison, LeFLS2 revealed only 36% and 40% amino acid identity with the LRR and the kinase domain, respectively, when aligned to EFR, which groups into the FLS2 subfamily LRR-XII. Therefore, LeFLS2 appears to be an appropriate candidate for the flagellin receptor in tomato. Genomic Southern blot analysis showed that *LeFLS2* is a single copy gene in tomato (Suppl. Fig. 3). Furthermore, sequence analysis of the *LeFLS2* genomic clone identified a small single intron following codon 1049, at the identical position as the intron in *AtFLS2* (data not shown).

Heterologous expression of LeFLS2

In order to examine whether LeFLS2 functions as a flagellin receptor, we stably introduced *LeFLS2* into the *A. thaliana* ecotype Ws-0, a natural occurring *fls2* mutant, and the T-DNA insertion line in Col-0 background *fls2* (Zipfel et al. 2004). The *LeFLS2* transcription was driven by the *AtFLS2* promoter to ensure similar expression levels and patterns compared with *AtFLS2*. We could establish several independent lines showing similar *LeFLS2* transcript accumulation as *AtFLS2* in Col-0 wild-type plants (Fig. 2A). We assayed these lines for their ability to bind radiolabeled flg22 peptides. None of the Ws/LeFLS2 and

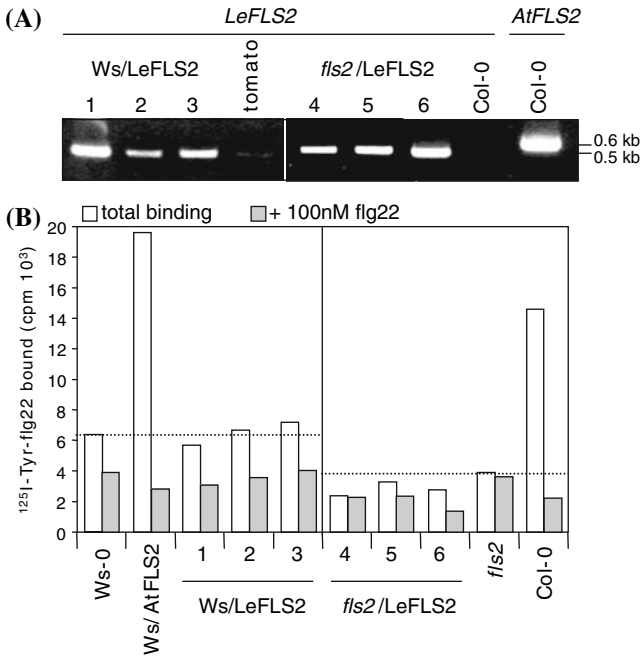


Fig. 2 Heterologous expression of *LeFLS2* in *Arabidopsis* Ws-0 and *fls2* lines. (**A**) RT-PCR of *LeFLS2* transcript accumulation in indicated lines (7) compared to its level and *AtFLS2* expression in control *Arabidopsis* (Col-0) and tomato plants, respectively. (**B**) Total binding and non-specific binding of ^{125}I-Tyr-flg22 (+100nM flg22) measured in homogenates of respective lines. Depicted are representative bars of two independent measurements. Dashed lines indicate background levels according to negative control lines Ws-0 and *fls2*; positive control lines are Ws/FLS2 and Col-0

fls2/LeFLS2 lines showed significant total flg22 binding above the control lines (Fig. 2B). When analyzing their responsiveness to flg22 and flg15 by measuring seedling growth, we were not able to detect any effect in the *LeFLS2* transgenic lines (Suppl. Fig. 4). This suggests that there are no functional flagellin binding sites present.

One possibility to explain these results is a lack of LeFLS2 protein expression or mis-localization. The well-characterized antibody recognizing AtFLS2 does not detect any signal in extracts of tomato, which can be explained by significant differences in the amino acid sequence of LeFLS2 and the AtFLS2 domain that has been used for immunization (Chinchilla et al. 2006). Therefore, we generated a *LeFLS2-GFP* fusion construct and stably transformed it into Ws-0 plants. By RT-PCR analysis we could clearly find transcript accumulation of the *LeFLS2-GFP* transgene (Fig. 3A). However, Western blot analysis using GFP-recognizing antibodies showed that the corresponding protein product was missing (Fig. 3B). We used ethylene production as an early response to flg22, and we tested these Ws/LeFLS2-GFP lines in this respect. None of them displayed increased ethylene levels above background (Fig. 3C). As positive controls we used Ws/FLS2-GFP lines that have been shown to fully complement the Ws-0 *fls2* mutant phenotype (Robatzek et al. 2006). Thus, we suggest that the LeFLS2 protein is either unstable, not expressed, or mis-targeted in *A. thaliana*. Integrity of the LeFLS2-GFP fusion construct was checked by biolistic bombardment of onion cells. Microscopy analysis clearly revealed GFP fluorescence at the cell periphery indicating plasma membrane localization (data not shown).

We analyzed *N. benthamiana*, which belongs to the family of *Solanaceae* and therefore is more closely related to tomato than *Arabidopsis*, for its potential to express LeFLS2 supplied by transient transformation. Western blot analysis using GFP-antibodies displayed clear signals for LeFLS2-GFP and AtFLS2-GFP transiently transformed leaves, whereas there were no signals in control (GUS) or untransformed samples (Fig. 4A). The band for LeFLS2-GFP indicates a molecular mass of ~200 kDa. The size of AtFLS2-GFP appeared slighter larger, which might be due to the additional triple myc-tag (Robatzek et al. 2006). Since the predicted molecular mass of LeFLS2 fused to GFP does not meet its detected size, it is very likely that LeFLS2 is similarly highly glycosylated as AtFLS2 (Chinchilla et al. 2006). Screening for potential N-linked glycosylation sites (NxS/T) revealed 17 putative sites for the LeFLS2- and 18 for the AtFLS2-LRR domain, of which 12 appear to be conserved among both (data not shown). It has been shown that nearly all predicted glycosylation sites in the Cf-9 receptor-like protein are linked to carbohydrates, and also contribute to Cf-9 function (van der Hoorn et al. 2005). Thus, glycosylation on multiple residues of the LeFLS2-LRR domain may be important for LeFLS2 expression and activity.

Chemical cross-linking experiments using ^{125}I-Tyr-flg22 revealed a major band at ~175 KDa in untransformed *N. benthamiana*, which was competed by an excess of unlabeled flg22 (Fig. 4B, right panel). This likely represents the endogenous flg22 binding sites of *N. benthamiana*, NbFLS2 (Hann and Rathjen 2007). An additional weaker band at ~150 kDa was also competed by unlabeled flg22. Most probably, this is a breakdown product of the major labeled band, as was also observed to occur for the EF-Tu binding site (Zipfel et al. 2006). Infiltration of *N. benthamiana* leaves with *A. tumefaciens* led to an increase in both, the ~175 kDa and the ~150 kDa band, which indicates that their expression might be induced (data not shown). When *N. benthamiana* was transiently transformed with *A. tumefaciens* either delivering AtFLS2-GFP or LeFLS2-GFP, an additional band of the size expected for these fusion products was detected in cross-linking experiments (Fig. 4B, left panels). Appearance of these bands was strongly suppressed in the presence of unlabeled flg22. Excess of unlabeled flg15, for which tomato exhibits a higher affinity than *Arabidopsis* (Chinchilla et al. 2006),

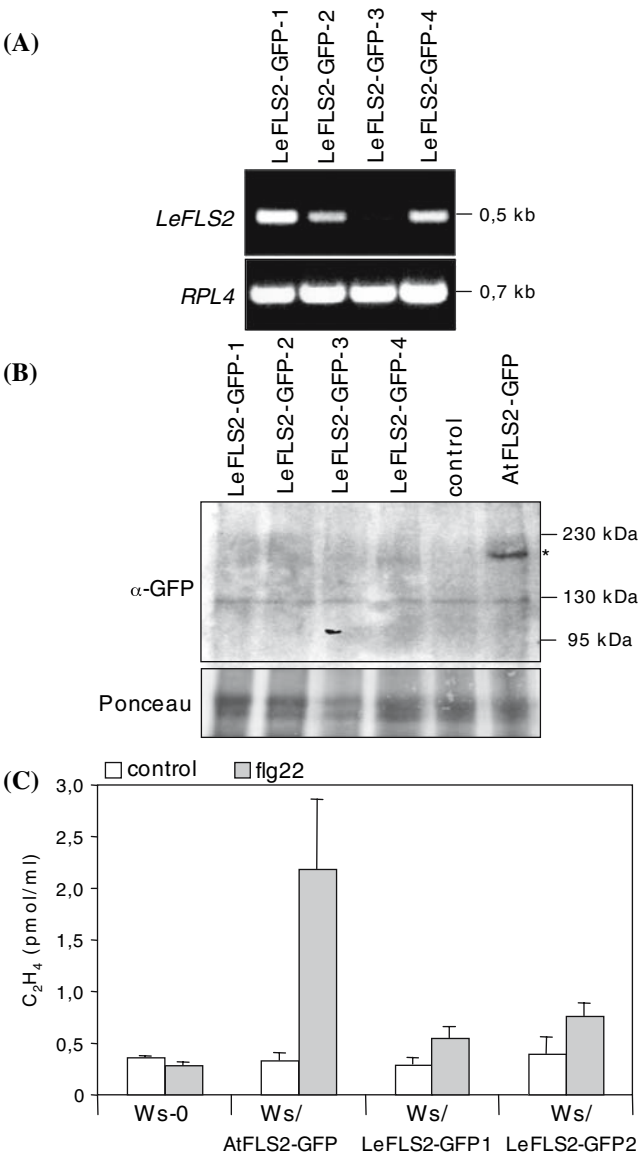


Fig. 3 Heterologous expression of LeFLS2-GFP in the *FLS2* deficient ecotype *Arabidopsis* Ws-0. (A) RT-PCR analysis of *LeFLS2-GFP* transgenic Ws-0 lines was performed using *LeFLS2*-specific primers. A control RT-PCR of *RPL4* indicates equal loading. (**B**) Western blot analysis was done on crude membrane extracts of the indicated Ws-0 transgenic lines revealed by GFP antibodies. The band corresponding to FLS2-GFP is labeled with an asterisk; all other bands appear to be background. Ponceau staining is shown for equal loading. (**C**) Ethylene production of leaf pieces after 4h incubation without or with 1 μM flg22 detected on the respective control and transgenic lines. Error bars indicate the standard deviation of four replicates

competed only the LeFLS2-GFP band efficiently. These data demonstrate that LeFLS2-GFP can be heterologously expressed in *N. benthamiana*, just like AtFLS2, and they provide evidence that LeFLS2-GFP constitutes a functional flg22 and flg15 binding site.

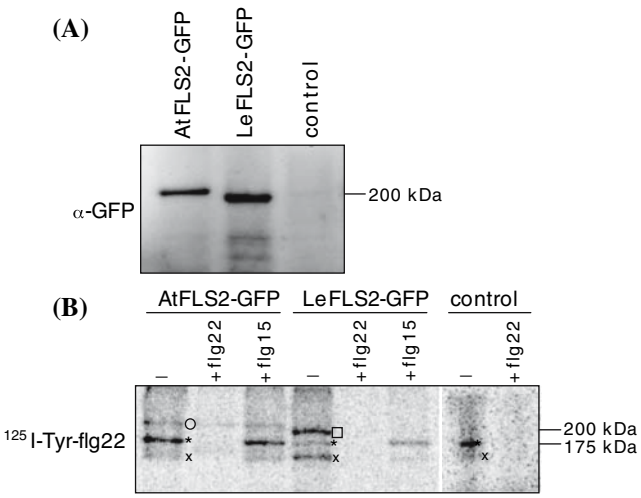


Fig. 4 Transient expression of LeFLS2-GFP in *N. benthamiana*. (A) Western blot analysis using crude membrane extracts of transformed *N. benthamiana* leaves mediated by *Agrobacterium*-infiltration of the indicated constructs using GFP-specific antibodies. (**B**) Cross-linking of ^{125}I-Tyr-flg22 detected in homogenates of respective transformed *N. benthamiana* leaves. Competition with 100nM unlabeled flg22 or flg15 is indicated. The flg22 binding site of AtFLS2-GFP is labeled with open circle, LeFLS2-GFP with open square, and those conferred by the endogenous flagellin perception system of *N. benthamiana* (control) with asterisk. Additionally bands of probably breakdown products are marked with crosses

Functional characterization of LeFLS2 as the flagellin receptor

Like most plant species, *N. benthamiana* is responsive to flg22. In order to utilize their capability to accumulate LeFLS2-GFP protein and at the same time apply functional assays for flagellin responses, we screened for potential characteristic differences in the flagellin peptide requirements between tomato and *N. benthamiana*. We found that both plant species generated ROS upon stimulation by flg22 and flg15 (Table 1). In contrast, using a flg15 peptide derived from the flagellin sequence of *E. coli*, flg15$^{E.coli}$, we observed a significant difference within both species. *N. benthamiana* plants were completely insensitive at concentrations tested, but tomato plants were fully reactive to flg15$^{E.coli}$ and showed a clear oxidative burst.

Making use of these species-specific differences in flagellin perception, we functionally tested LeFLS2-GFP transiently transformed in *N. benthamiana* leaves. Our oxidative burst data clearly show that only *N. benthamiana* leaves expressing LeFLS2-GFP were responsive to flg15$^{E.coli}$, while samples from control transformed plants and plants transiently transformed with AtFLS2-GFP showed no reaction to this peptide (Fig. 5A). Similarly, a flg15$^{E.coli}$ treatment triggered production of ethylene only in the LeFLS2-GFP expressing *N. benthamiana* (Fig. 5B). Control transformed leaves (GUS and AtFLS2) were able

Table 1 Species-specific difference in the responsiveness to flagellin-derived peptides between tomato and *N. benthamiana*. Accumulation of reactive oxygen species (ROS) in tomato (Le) and *N. benthamiana* (Nb) upon addition of 1 μM of the indicated flagellin-derived peptides

	None	flg22	flg15	$flg15^{E.coli}$
Le	–	+	+	+
Nb	–	+	+	–

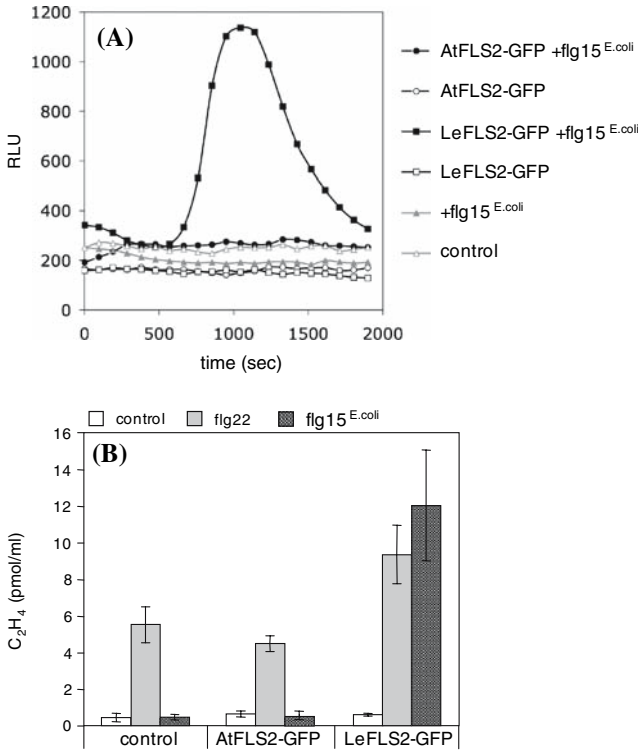


Fig. 5 Heterologous expression of LeFLS2-GFP in *N. benthamiana* leads to the presence of a flagellin perception system with the characteristics of tomato. (**A**) Oxidative burst measured on leaf pieces of transiently transformed *N. benthamiana* leaves with the indicated constructs triggered by 1 μM $flg15^{E.coli}$. For negative control a GUS construct has been used. ROS generation is given in RLU as emitted by luminescence. (**B**) Ethylene content of leaf pieces after 4h incubation without or with 1 μM of each indicated flagellin peptide detected in respective transiently transformed *N. benthamiana* leaves. Error bars indicate the standard deviation of four replicates

to respond to flg22, but not to $flg15^{E.coli}$. In summary, *N. benthamiana* gained the flagellin perception system characteristic for tomato when LeFLS2 expression was provided. We therefore conclude that the isolated and cloned sequence *LeFLS2* encodes the flagellin receptor of tomato.

Discussion

LeFLS2 accumulation is compromised in *A. thaliana*

In our study, we identified the tomato flagellin receptor LeFLS2, which belongs to the family of plant LRR-RLKs and is highly homologous to AtFLS2. To test whether LeFLS2 indeed acts as a functional flagellin receptor, heterologous complementation lines in *A. thaliana* were generated. However, none of these lines showed accumulation of LeFLS2. Generally, transmembrane proteins are delivered across the endoplasmatic reticulum (ER) via the secretory pathway. One possible explanation could be that the LeFLS2 signal peptide is not recognized in *A. thaliana*. In addition, mis-folded and non-functional proteins do not enter the secretory pathway and are discarded by the so-called quality control system, which is also known as ER-associated protein degradation (ERAD) (Lord et al. 2000; Pimpl et al. 2006). Reminiscent of TLRs, according to the structural model of TLR3, plant flagellin receptor LRR domains are likely to be masked by carbohydrates (Choe et al. 2005). These N-linked glycosylation is not only essential for proper protein folding, but also its degree and type serve as a mark for the protein folding status by which the quality control mechanism distinguishes mature proteins from mis-folded ones (Helenius and Aebi 2004). Although most of the potential glycosylation sites are shared between AtFLS2 and LeFLS2, the few differences could trigger LeFLS2 for ERAD in *A. thaliana*. It could be shown for Cf-9 that removal of one glycosylation site in the LRR domain resulted in a loss of protein accumulation (van der Hoorn et al. 2005). In addition, certain amino acid differences between both flagellin receptors may affect molecular interactions with chaperones important for quality control (Bukau et al. 2006). Mutation of the HSP90 chaperone was reported to decrease abundance of the intracellular immune receptor RPM1 (Hubert et al. 2003). Furthermore, it has been demonstrated that mutations of the barley powdery mildew resistance protein MLO affect its protein stability by targeting it to the ERAD pathway (Muller et al. 2005).

Previously studied *fls2 Arabidopsis* mutants revealed that *fls-24* and *fls2–17* are impaired in flg22 binding, the first mutated in the LRR domain, and latter containing a G to R point mutation at position 1064 in its kinase domain (Gomez-Gomez and Boller 2000; Gomez-Gomez et al. 2001). Such mutations within the kinase activation loop result in a loss of autophosphorylation activity as have been seen with *fls2–17*, *clv1–2* and *bri1–101* (Gomez-Gomez et al. 2001; Dievart and Clark 2003). We discovered that in contrast to *fls2–24*, the *fls2–17* mutant

lacks AtFLS2 protein accumulation (Suppl. Fig. 5A). It appears that this is the reason why this mutant fails to bind flg22 (Gomez-Gomez et al. 2001). Moreover, the *fls2–17* point mutation was re-created in the FLS2^{G1064} variant fused to GFP and introduced into Ws-0 plants. Independent lines exhibiting *FLS2^{G1064}-GFP* mRNA accumulation were selected and shown to not accumulate respective FLS2^{G1064}-GFP protein (data not shown and Suppl. Fig. 5B). We propose that biologically inactive AtFLS2 is subjected to degradation mediated by the plant's quality control system. Supporting our results, autophosphorylation of Xa21 is mandatory for protein stability (Xu et al. 2006). Furthermore, findings in yeast show that cytoplasmic domains of transmembrane proteins are critical for directing them to ERAD (Meusser et al. 2005).

Expression of LeFLS2 confers a specific flagellin perception system characteristic for tomato in *N. benthamiana*

In animals, species-specific differences have been described for the TLR response to RNA (Heil et al. 2004). Similarly, different plants display specific characteristics for their respective flagellin perception systems (Bauer et al. 2001; Chinchilla et al. 2006). We found that there are not only differences in the recognition of the flagellin epitope between plant families, but also between species in the same family. The *solanaceous* tomato and *N. benthamiana* contain both a highly sensitive perception system for the 22 and 15 amino acid peptides derived from the *P. syringae* flagellin, whereas the 15 amino acid peptide derived from *E. coli* flagellin was only highly active in tomato. *N. benthamiana* expressing LeFLS2 gained responsiveness towards flg15$^{E.coli}$, and thereby acquired the flagellin perception system specific for tomato. During the past, several flagellin peptide variants have been discovered that do not elicit defense responses in *A. thaliana* (Bauer et al. 2001; Pfund et al. 2004; Sun et al. 2006). Furthermore, the so-called broad-species active flg22 peptide did not trigger defense responses in rice cell cultures, whereas they are responsive to a specific flagellin from the incompatible strain *P. avenae* (Felix et al. 1999; Che et al. 2000). It remains an open question what selective forces drive evolution of PAMP receptors such as the flagellin receptor into different directions.

Acknowledgements We thank Dr. Helge Sierotzki (Syngenta, Switzerland) for kindly providing tomato plants variety ''Roter Gnom'' and Heidrun Häweker for technical support. This work was supported by DFG to S.R. (SFB670), and by a grant of the Swiss National Science Foundation to T.B.

References

Akira S, Takeda K (2004) Toll-like receptor signaling. Nat Rev Immunol 4:499–511

Asai T, Tena G, Plotnikova J et al (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415:977–983

Bauer Z, Gomez-Gomez L, Boller T, Felix G (2001) Sensitivity of different ecotypes and mutants of Arabidopsis thaliana toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. J Biol Chem 276:45669–45676

Bukau B, Weissmann J, Horwich A (2006) Molecular chaperones and protein quality control. Cell 125:443–451

Che FS, Nakajima Y, Tanaka N, Iwano M, Yoshida T, Takayama S, Kadota I, Isogai A (2000) Flagellin from an incompatible strain of Pseudomonas avenae induces a resistance response in cultured rice cells. J Biol Chem 275:32347–32356

Chinchilla D, Bauer Z, Regenass M, Boller T, Felix G (2006) The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 18:1–12

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124:803–814

Choe J, Kelker MS, Wilson IA (2005) Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science 309:581–585

Dievart A, Clark SE (2003) Using mutant alleles to determine the structure and function of leucine-rich repeat receptor-like kinases. Curr Opin Plant Biol 6:507–516

Dievart A, Clark SE (2004) LRR-containing receptors regulating plant development and defense. Development 131:251–261

Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 18:265–276

Felix G, Grosskopf DG, Regenass M, Boller T (1991) Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells. Proc Natl Acad Sci USA 88:8831–8834

Gomez-Gomez L, Bauer Z, Boller T (2001) Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in Arabidopsis. Plant Cell 13:1155–1163

Gomez-Gomez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol Cell 5:1003–1011

Gomez-Gomez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. Plant J 18:277–284

Hann DR, Rathjen JP (2007) Early events in the pathogenicity of Pseudomonas syringae on Nicotiana benthamiana. Plant J 49: 607–618

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303:1526–1529

Helenius A, Aebi M (2004) Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem 73:1019–1049

Hubert DA, Tornero P, Belkhadir Y, Krishna P, Takahashi A, Shirasu K, Dangl JL (2003) Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein. EMBO J 22:5679–5689

Kobe B, Kajava AV (2001) The leucine-rich repeat as a protein recognition motif. Curr Opin Struct Biol 11:725–732

Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell 16:3496–3507

Lord JM, Davey J, Frigero L, Roberts LM (2000) Endoplasmic reticulum-associated protein degradation. Semin Cell Dev Biol 11:159–164

Medzhitov R, Janeway CA Jr (2002) Decoding the patterns of self and nonself by the innate immune system. Science 296:298–300

Meindl T, Boller T, Felix G (2000) The bacterial elicitor flagellin activates its receptor in tomato cells according to the address-message concept. Plant Cell 12:1783–1794

Meusser B, Hirsch C, Jarosch E, Sommer T (2005) ERAD: the long road to destruction. Nat Cell Biol 7:766–772

Montoya T, Nomura T, Farrar K, Kanata T, Yokota T Bishop GJ (2002) Cloning the tomato curl3 gene highlights the putative dual role of the leucine-rich repeat receptor kinase tBRI1/SR160 in plant steroid hormone and peptide hormone signaling. Plant Cell 14:3163–3176

Muller J, Piffanelli P, Devoto A, Miklis M, Elliot C, Ortmann B, Schulze-Lefert P, Panstruga R (2005) Conserved ERAD-like quality control of a plant polytopic membrane protein. Plant Cell 17:149–163

Nuhse TS, Peck SC, Hirt H, Boller T (2000) Microbial elicitors induce activation and dual phosphorylation of the Arabidopsis thaliana MAPK 6. J Biol Chem 275: 7521–7526

Pfund C, Tans-Kersten J, Dunning FM, Alonso JM, Ecker JR, Allen C, Bent AF (2004) Flagellin is not a major defense elicitor in Ralstonia solanacearum cells or extracts applied to Arabidopsis thaliana. Mol Plant Microbe Interact 17:696–706

Pimpl P, Taylor JP, Snowden C, Hillmer S, Robinson DG, Denecke J (2006) Golgi-mediated vacuolar sorting of the endoplasmic reticulum chaperone BiP may play an active role in quality control within the secretory pathway. Plant Cell 18:198–211

Rechsteiner M, Rogers SW (1996) PEST sequences and regulation by proteolysis. Trends Biochem Sci 21:267–271

Robatzek S, Chinchilla D, Boller T (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. Genes Dev 20:537–542

Ron M, Avni A (2004) The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. Plant Cell 16:1604–1615

Shiu SH, Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA 98:10763–10768

Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, Cookson BT, Aderem A (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 4:1247–1253

Sun W, Dunning FM, Pfund C, Weingarten R, Bent AF (2006) Within-species flagellin polymorphism in Xanthomonas campestris pv campestris and its impact on elicitation of Arabidopsis FLAGELLIN SENSING2-dependent defenses. Plant Cell 18:764–779

van der Hoorn RA, Wulff BB, Rivas S, Durrant MC, van der Ploeg A, de Wit PJ, Jones JD (2005) Structure-function analysis of cf-9, a receptor-like protein with extracytoplasmic leucine-rich repeats. Plant Cell 17:1000–1015

Vert G, Nemhauser JL, Geldner N, Hong F, Chory J (2005) Molecular mechanisms of steroid hormone signaling in plants. Annu Rev Cell Dev Biol 21:177–201

Walker JC (1994) Structure and function of the receptor-like protein kinases of higher plants. Plant Mol Biol 26:1599–1609

Xu WH, Wang YS, Liu GZ, Chen X, Tinjuangjun P, Pi LY, Song WY (2006) The autophosphorylated Ser686, Thr688, and Ser689 residues in the intracellular juxtamembrane domain of XA21 are implicated in stability control of rice receptor-like kinase. Plant J 45:740–751

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 125:749–760

Zipfel C, Robatzek S, Navarro L, Oakeley E, Jones JDG, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428:764–767